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A variant of *Yarrowia lipolytica* lipase with improved activity and enantioselectivity for resolution of 2-bromo-arylacetic acid esters

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ABSTRACT

A variant of Lip2p lipase from *Yarrowia lipolytica* yeast was used for the resolution of 2-bromophenyl and *o*-tolyl acid esters, an important class of chemical intermediates for the pharmaceutical industry. In comparison with wild-type Lip2p, this variant, which contains one single amino acid change in the active site of the enzyme, V232A, displayed an enantioselectivity enhanced by one order of magnitude for the resolution of 2-bromo-phenylacetic acid ethyl ester (*E*-value increased from 5.5 to 59 for wild-type and V232A, respectively) and by fourfold for the resolution of 2-bromo-*o*-tolylacetic acid ethyl ester (going from an *E*-value of 27 to 111 for the wild-type and V232A, respectively). A remarkable increase in reaction velocity was also observed for both compounds, as a result of a significant gain in reactivity towards the favoured (*S*)-enantiomer (3- and 16-fold increase for 2-bromo-phenylacetic and -*o*-tolylacetic acid ethyl esters, respectively). These results demonstrate the key role of the V232 amino acid in enantiomer recognition and selectivity.

1. Introduction

The market for drugs sold in single-enantiomer form is expanding rapidly (13% increase in 2005) and represented $205 billion worldwide in 2005.[1] It concerns the fields of pharmaceutical, agricultural and synthetic organic chemistry. Enantiopure carboxylic acids are important building blocks for the synthesis of many pharmaceuticals, pesticides and natural compounds such as pheromones. 2-Arylpropionic acids have the properties of non-steroid anti-inflammatory drugs (Ibuprofen, Naproxen, Ketoprofen)[2–5] whereas 2-halogeno-carboxylic acids are important intermediates found in synthetic pathways of a number of drugs, such as prostaglandin, prostacyclin, semi-synthetic penicillin and thiazolium salts.[6–12] For instance, ethyl ester derivatives of 2-bromo-*o*-tolylacetic acid are used as precursors for the synthesis of analgesics and non-peptide angiotensin II-receptor antagonists.[6–8]

Over the past decades, enzymes have become important tools for racemic resolution and have found more and more use, in competition with chemical asymmetric synthesis, stereoselective crystallization and chiral chromatography.

In this field, lipases are the most frequently used biocatalysts. The reasons for such interest lie in their high stability, their non-requirement for co-factors, and mostly their synthetic activity in organic solvents. Moreover, lipases are also capable of catalyzing reactions with non-natural substrates.

In a previous paper,[13] we demonstrated that Lip2p lipase, which is extra-cellularly produced by the non-pathogenic lipolytic *Yarrowia lipolytica* yeast, is an efficient enzyme for the resolution of 2-halogeno-carboxylic ethyl esters via transesterification with 1-octanol in *n*-octane. The resolution of 2-bromo-*p*-tolylacetic acid ethyl ester using Lip2p revealed an enantioselectivity of 28, comparable to the value obtained with *Burkholderia cepacia* lipase (*E*-value = 30), the most efficient lipase described so far in the literature.[6,7] More interestingly, *Y. lipolytica* Lip2p was shown to be the only known enzyme able to catalyze the resolution of 2-bromo-*o*-tolylacetic acid ethyl ester with an *E*-value of 27. While Lip2p indicated a clear preference for the (*S*)-enantiomer, *B. cepacia* lipase was rather selective for the (*R*)-enantiomer. In addition, a remarkable increase in velocity was observed using *Y. lipolytica* Lip2p compared to *B. cepacia* lipase (69- and 26-fold higher for *para*- and *ortho*-substituted substrates, respectively). Nonetheless, in spite of the interesting performances of *Y. lipolytica* Lip2p, *E*-values remain insufficient for industrial requirements and thus the need for a more selective catalyst appeared crucial. The evolution of Lip2p was considered using site-directed mutagenesis to search

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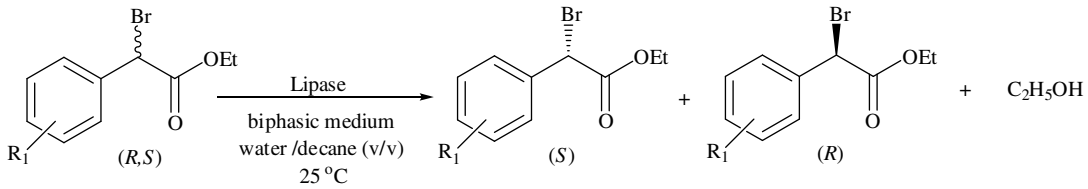

2-bromo-phenylacetic acid ethyl esters: R_1 = H
2-bromo-*o*-tolylacetic acid ethyl esters: R_1 = *ortho*-CH_3

Scheme 1.

for variants with enhanced activity and selectivity towards the resolution of 2-halogeno-carboxylic esters.

Herein we report the characterization of one particular variant of *Y. lipolytica* Lip2p isolated from a previous library screen,[14] for the resolution of 2-halogeno-carboxylic esters. In comparison to wild-type Lip2p, this variant contains one single amino acid change in the active site, V232A, which remarkably led to an increase in enantioselectivity for the resolution of 2-bromo-phenylacetic acid ethyl ester.

2. Results and discussion

Performances of wild-type *Y. lipolytica* Lip2p and its variant V232A were first investigated from the point of view of their hydrolytic activity towards *para*-nitrophenol butyrate and then for their enantioselectivity towards the hydrolysis of 2-bromo-phenyl- and 2-bromo-*o*-tolyl- acetic acid esters in a biphasic system (water/organic solvent) (Scheme 1).

2.1. Production and quantification of Lip2p and its V232A variant

Given the absence of crystallographic data on the Lip2p lipase, a three-dimensional model of Lip2p was predicted by homology modelling using as templates the X-ray structures of very similar lipases.[14] On the basis of this model, five amino acids forming the hydrophobic substrate binding site of Lip2p were targeted by site-directed mutagenesis. Among the library of Lip2p variants which were constructed, one particular variant V232A revealed very promising properties. This variant, together with the wild-type Lip2p, was first screened for their hydrolytic activity of the *para*-nitrophenol butyrate. Results obtained for both enzymes are shown in Table 1. Three independent clones of the two strains were cultivated to produce the wild-type enzyme and its variant. Standard deviations of a comparable order were obtained for all three clones of each enzyme, indicating that all clones own a single copy of the lipase gene, introduced at the zeta docking platform at the *LEU2* locus in the genome,[15] leading to good reproducibility of the enzyme expression system and thus enabling a comparison of enzyme activities. In comparison to wild-type Lip2p, variant V232A displayed an activity reduced to about 80% of the wild-type Lip2p activity (66 and 83 μmol min^{-1} mL^{-1} for V232A and wild-type Lip2p, respectively). Nonetheless, results indicate that the replacement of Val232 with a smaller hydrophobic alanine amino acid did not significantly affect the enzyme activity.

Table 1
Comparison of wild-type *Y. lipolytica* lipase and its variant V232A during hydrolysis of *para*-nitrophenol butyrate at 25 °C

Lipase	Clones	Initial rate[a] (μmol min^{-1} mL^{-1})	Average initial rate (μmol min^{-1} mL^{-1})
Wild-type	1	87.1 ± 6.0	83.2 ± 4.7
	2	80.3 ± 1.9	
	3	82.2 ± 3.5	
Variant V232A	1	68.0 ± 3.6	66.3 ± 3.1
	2	64.0 ± 2.0	
	3	67.0 ± 3.0	

[a] Activity assays were realized in triplicates.

2.2. Resolution of (*R,S*)-2-bromo-phenylacetic acid ethyl ester

Both enzymes, wild-type Lip2p and its V232A variant, were then evaluated for their hydrolytic activity on a racemic mixture of 2-bromo-phenylacetic acid ethyl ester in a biphasic medium (water/decane v/v) at 25 °C (Fig. 1). Results indicate a remarkable 10-fold increase in enantioselectivity for the V232A variant, going from an *E*-value of 5.5 to 59 for wild-type Lip2p and V232A variant, respectively (Table 2). In particular, the V232A variant displayed a 2.8-fold enhanced activity towards the (*S*)-enantiomer whereas its activity towards the (*R*)-enantiomer is about 3.9 times lower than results obtained using wild-type Lip2p. While an increase in enzyme selectivity is often accompanied by a decrease in velocity, no such effect has been observed in the case of the V232A variant. Indeed, the resolution of (*R,S*)-2-bromo-phenylacetic acid ethyl ester using the V232A variant is obtained within a shorter time that is needed when using wild-type Lip2p, which is an advantage for the use of this enzyme in an industrial process. It should be noted that the enantioselectivity determined for the hydrolysis reaction using the wild-type enzyme is equivalent to the value previously determined for the transesterification reaction.[13] Such results might thus indicate that the selectivity is controlled by the first step of the catalysis, namely the formation of the tetrahedral intermediate. Enantiomeric excesses of the substrate and the product are, respectively, 99.9% and 84% at 54.3% total conver-

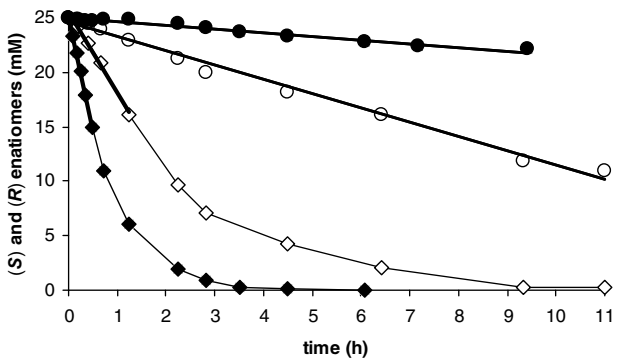

Figure 1. Hydrolysis kinetics versus *R,S*-2-bromo-phenylacetic acid ethyl ester in a biphasic medium (water/decane v/v) at 25 °C. Wild-type lipase activity versus *S*- (◇) and *R*- (○) enantiomers. Variant V232A lipase activity versus *S*- (♦) and *R*- (●) enantiomers. Thick lines represent initial velocity.

Table 2
Comparison of activity and selectivity of wild-type *Y. lipolytica* Lip2p lipase and its variant V232A during hydrolysis of (*R*,*S*)-2-bromo-phenylacetic acid ethyl ester racemate

	(*S*)-initial rate (μmol h^{-1} mL^{-1})	(*R*)-initial rate (μmol h^{-1} mL^{-1})	*E*-value (vi*R*/vi*S*)[a]	Conversion (%)	ee_s[b] (%)	ee_p[c] (%)
Wild-type lipase	7.24	1.32	5.5	55.1 (4.5 h)	61.9	50.5
Variant V232A	20.3	0.34	59	54.3 (6 h)	99.9	84

[a] vi*R*, vi*S*: initial rates.
[b] Substrate enantiomeric excess.
[c] Product enantiomeric excess.

sion after 6 h of reaction. At this point in time, 91.3% of the (*R*)-enantiomer can be recovered with a purity of 99.0%, and 97.1% at 92.0% purity for the (*S*)-enantiomer.

2.3. Resolution of (*R*,*S*)-2-bromo-*o*-tolylacetic acid ethyl ester

A comparable study was carried out for the resolution of a racemic mixture of 2-bromo-*o*-tolylacetic acid ethyl esters using wild-type Lip2p lipase and its V232A variant. Kinetics are shown in Figure 2 while activity and selectivity parameters are given in Table 3.

The presence of a methyl group at the *ortho*-position of the aromatic ring, causes steric hindrance, clearly inhibiting the kinetics. The activity of wild-type Lip2p was 25.6 times lower for the resolution of (*R*,*S*)-2-bromo-*o*-tolylacetic acid ethyl esters than results

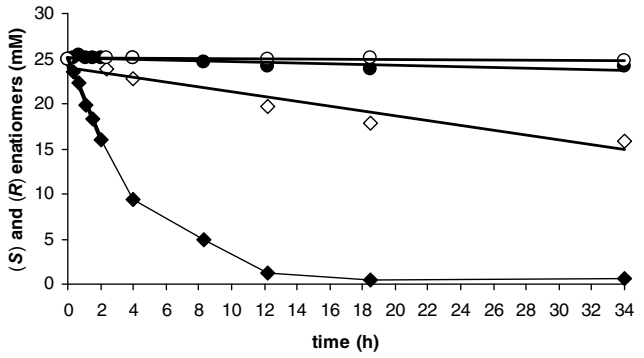

Figure 2. Hydrolysis kinetics versus 2-bromo-*o*-tolylacetic acid ethyl esters in a biphasic medium (water/decane v/v) at 25 °C. Wild-type lipase activity versus *S*- (◇) and *R*- (○) enantiomers. Variant V232A lipase activity versus *S*- (◆) and *R*- (●) enantiomers. Thick lines represent initial velocity.

observed for the resolution of (*R*,*S*)-2-bromo-phenylacetic acid ethyl esters in which there is no substitution on the aromatic ring. As observed for (*R*,*S*)-2-bromo-phenylacetic acid ethyl esters, the non-substituted substrate, the change of valine 232 into an alanine also led to a 10-fold increase in activity towards the preferred (*S*)-enantiomer of 2-bromo-*o*-tolylacetic acid ethyl ester. The velocity ratio between the two substrates was drastically reduced from 25.6 to 4.5 (comparison of values given in Tables 2 and 3). For the transesterification reaction with octanol, an *E*-value of 27 was found for the wild-type enzyme.[13] A similar *E*-value is also obtained for the hydrolysis reaction, suggesting again that discrimination between the (*R*,*S*)-enantiomers might occur during the first catalytic step of formation of the tetrahedral intermediate. The enantioselectivity of the variant V232A is increased four times (going from 27 to 111). In this case, improvement is exclusively due to the better catalysis of the preferred (*S*)-enantiomer (16 times higher), although catalysis of poorly recognized *R*-enantiomer is also found to be increased by fourfold. After 18.5 h, the conversion is 51.3% with enantiomeric excesses of 96.1 and 91.2 for substrate and product, respectively. Also, 95.6% of the (*R*)-enantiomer can be recovered with a purity of 98.1%, and 98.1% at 95.6% purity for the (*S*)-enantiomer.

2.4. Influence of the ester group on the activity and selectivity

For both substrates, the influence of the nature of the ester chain on the activity and enantioselectivity of variant V232A was investigated (Tables 4 and 5). It is noteworthy that results obtained for the ethyl- and the octyl-esters of 2-bromo-phenylacetic acid are quite similar. Indeed, for both substrates, an increase was observed in the catalysis of the preferred (*S*)-enantiomer while a decrease was observed for the catalysis of the poorly reacting (*R*)-enantiomer, thus leading to a slight augmentation of the

Table 3
Comparison of activity and selectivity of wild-type *Y. lipolytica* lipase and its variant V232A during hydrolysis of (*R*,*S*)-2-bromo-*o*-tolylacetic acid ethyl ester racemate

	(*S*)-initial rate (μmol h^{-1} mL^{-1})	(*R*)-initial rate (μmol h^{-1} mL^{-1})	*E*-value (vi*R*/vi*S*)[a]	Conversion (%)	ee_s[b] (%)	ee_p[c] (%)
Wild-type lipase	0.282	0.0105	27			
Variant V232A	4.5	0.040	111	51.3 (18.5 h)	96.1	91.2

[a] vi*R*, vi*S*: initial rates.
[b] Substrate enantiomeric excess.
[c] Product enantiomeric excess.

Table 4
Comparison of the hydrolysis kinetic of ethyl-, octyl- and benzyl-esters of 2-bromo-phenylacetic acid by the variant V232A

	(*S*)-initial rate (μmol h^{-1} mL^{-1})	(*R*)-initial rate (μmol h^{-1} mL^{-1})	*E*-value (vi*R*/vi*S*)[a]	Conversion (%)	ee_s[b] (%)	ee_p[c] (%)
Ethyl ester	20.3	0.34	59	54.3 (6 h)	99.89	84
Octyl ester	23	0.32	72	54.6 (7 h)	99.94	83.1
Benzyl ester	39	2.14	18	55.6 (1.33 h)	98.2	78.4

[a] vi*R*, vi*S*: initial rates.
[b] Substrate enantiomeric excess.
[c] Product enantiomeric excess.

Table 5
Comparison of the hydrolysis kinetic of ethyl, octyl and benzyl esters of 2-bromo-*o*-tolylacetic acid by the variant V232A

	(*S*)-initial rate (μmol h^{-1} mL^{-1})	(*R*)-initial rate (μmol h^{-1} mL^{-1})	*E*-value (vi*R*/vi*S*)[a]	Conversion (%)	ee_s[b] (%)	ee_p[c] (%)
Ethyl ester	4.5	0.04	111	51.3 (18.5 h)	96.1	91.2
Octyl ester	4.9	0.10	49	54 (22 h)	95.9	81.8
Benzyl ester	10.3	0.88	12	55.6 (1.33 h)	98.2	78.4

[a] vi*R*, vi*S*: initial rates.
[b] Substrate enantiomeric excess.
[c] Product enantiomeric excess.

enantioselectivity value. Conversely, the enantioselectivity was reduced for esters of 2-bromo-*o*-tolylacetic acid, due to a better catalysis of the (*R*)-enantiomer. On the other hand, the use of benzyl-esters of both 2-bromo-phenylacetic acid and 2-bromo-*o*-tolylacetic acid led to increased rates of hydrolysis (Tables 4 and 5). Indeed, a velocity increased by twofold was obtained for the (*S*)-enantiomer of both substrates, whereas catalysis of the (*R*)-enantiomer is increased by 6.5-fold for the bromo-phenylacetic acid (Table 4) and by 10-fold for 2-bromo-*o*-tolylacetic acid (Table 5). As a consequence, the enantioselectivity was lowered to a value of 18 and 12 for 2-bromo-phenylacetic acid ester and 2-bromo-*o*-tolylacetic acid ester, respectively. The same results were obtained with the wild-type enzyme (data not shown).

3. Conclusion

In conclusion, we have demonstrated that variant V232A of *Y. lipolytica* lipase is a very active and selective catalyst for the hydrolysis of 2-bromo-phenyl and tolyl acetic acid ethyl esters. The change of a single amino acid located in the active site of the enzyme led to a drastic 10-fold increase in enantioselectivity for the resolution of (*R*,*S*)-2-bromo-phenylacetic acid ethyl ester racemates. Enantioselectivity was also increased for the resolution of (*R*,*S*)-2-bromo-*o*-tolylacetic acid ethyl ester racemates, reaching a value of 111. Moreover, the gain in enantioselectivity for both substrates is accompanied by an increase in the catalysis rate of the preferred (*S*)-enantiomer.

The activity and enantioselectivity displayed by the variant V232A of *Y. lipolytica* lipase are compatible with the industrial use of this enzyme for the production of pharmaceutical drugs.

We are currently working on the determination of the crystallographic structure of this lipase in order to understand, on a molecular level, the structural determinants controlling Lip2p enantioselectivity and the effects induced by V232A mutation on the enantio-discrimination of 2-bromo phenyl and *o*-tolyl acid esters. In parallel, saturation mutagenesis techniques, consisting of changing the residue with all the others amino acids, will be used to help better understand the enantiopreference phenomenon and to improve further performances of this promising enzyme.

4. Materials and methods

4.1. Biological reagents

Lip2p lipase from *Y. lipolytica* and its variant were produced from a strain of *Y. lipolytica* (JMY 1212) as described elsewhere.[15] The oleic acid-inducible *POX2* promotor was used to drive transcription of the *LIP2* gene. Protein secretion is directed by the wild-type lipase targeting sequence. For growth in a tube, 5 mL YPD medium (yeast extract 10 g/L, peptone 10 g/L and glucose 10 g/L) is used. This pre-culture is used to inoculate a 250 mL erlen flask containing 50 mL YTO medium (yeast extract 10 g/L, peptone 20 g/L and oleic acid 20 g/L) in a 100 mM phosphate buffer (pH 6.8). Stock preparation of oleic acid (200 g of oleic acid/L, 5 g of Tween 40/L) is subjected to sonication three times for 1 min on ice for emulsification purposes. After centrifugation and 0.2 μm filtration, the enzyme is directly used for enzymatic assays.

4.2. Chemical reagents

Peptone, tryptone and yeast extract were purchased from Difco (Difco, Paris, France).

All chemicals were of commercial quality and were purchased from Sigma/Aldrich. *n*-Decane was dried over molecular sieves (3 Å) before use.

4.3. General procedure for the preparation of 2-bromo carboxylic acid esters

The procedure for the preparation of (±)-2-bromo-phenylacetic acid ethyl and octyl ester, (±)-2-bromo-*o*-tolylacetic acid ethyl octyl and benzyl esters was described in a previous paper.[13]

4.4. Procedure for enzymatic reactions

4.4.1. Hydrolysis of *p*-nitrophenyl butyrate

The lipase activity in the culture supernatant was determined by monitoring the hydrolysis of *p*-nitrophenyl butyrate (*p*NPB) into butyrate and *p*-nitrophenol.[16] The method was optimized using 2-methyl-butan-2-ol (2M2B) as solvent to solubilize *p*-nitrophenyl butyrate. Lipase activity was measured in 96-well microplates with 20 μL of the supernatant containing Lip2, 175 μL of a 100 mM phosphate buffer, pH 7.2, 100 mM NaCl and 5 μL of *p*NPB 40 mM in 2M2B. Activity was measured by following absorbance at 405 nm at 25 °C for 10 min using the VersaMax tunable microplate reader apparatus (Molecular Devices, Rennes, France). One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per min at 25 °C and pH 7.2.

4.4.2. Hydrolysis of 2-bromo phenyl and tolyl acetic acid esters

Hydrolysis was carried out in 1.5 mL eppendorf tubes containing a biphasic medium composed of 0.5 mL dried decane containing the ester (100 mM) and 0.5 mL of the aqueous enzymatic solution. The mixture was shaken in a Vortex Genie 2 (D. Dutscher, Brumat, France). Reactions were realized at 25 °C. At regular time intervals, the progress of the reaction was followed by taking samples after phase separation by centrifugation (100 μL diluted in 1 mL hexane).

4.5. HPLC analysis

The HPLC device was equipped with a chiral column: Chiralpack OJ (25 cm × 4.6 mm) (Daicel Chemical Industries Ltd, Japan) connected to a UV detector (at 254 nm). A flow rate of 1.0 mL/min and a 40 °C column temperature were used. The mobile phase was composed of a mixture of *n*-hexane/isopropanol (70:30 v/v) for all the esters, except for (±)-2-bromo-*o*-tolyl octyl acetate where a mixture of *n*-hexane/isopropanol (98:2 v/v) was used.

4.6. Determination of the enantiomeric excess (ee), conversion and enantioselectivity (*E*)

From HPLC results, enantiomeric excess (ee) was calculated as defined below: $ee_s = \{[R] - [S]\}_s/\{[R] + [S]\}_s$ (s = substrate) and the conversion: $C = 1 - [(R-S)_t/(R-S)_{t=0}] * 100$.

The enantioselectivity value was the ratio of the initial rate of (*S*)-enantiomer production (vi*R*) to the initial rate of (*R*)-enantiomer production (vi*S*): $E = (viS/viR)$. The initial rates were determined by linear regression, which is indicated in Figures 1 and 2.

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